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Agriculture Development Fund

FINAL REPORT

19980065

A NEW APPROACH ON THE CONTROL OF PRE-HARVEST SPROUTING IN CPS WHITE WHEAT AND MALTING BARLEY: THE ROLE OF AQUAPORIN GENES

Funded by
The Agriculture Development Fund

May 2002
Prepared by: University of Saskatchewan

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A New Approach on the Control of Pre-Harvest Sprouting in CPS White Wheat and Malting Barley: The Role of Aquaporin Genes

Final Report

ADF-9800065

January 31, 2002

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Final Report: A New Approach on the Control of Pre-Harvest Sprouting in CPS White Wheat and Malting Barley: The Role of Aquaporin Genes

(a) Summary

Fourteen CPS wheat lines, 7 pre-harvest sprouting sensitive and 7 resistance lines were tested for germination and the presence of aquaporins at different stages of maturity. Northern blot analysis clearly showed earlier expression of four aquaporins genes in pre-harvest sprouting sensitive lines indicating they may play a role in pre-harvest sprouting. Pre-wetted barley seeds stored at 40°C, 85% RH deteriorated faster than seeds stored at 35°C while faster than at 30°C. Seeds stored under a N_2 atmosphere deteriorate faster than seeds stored in air suggesting that seeds deteriorate due to anoxia. This is the result of seeds absorbing a small amount of water (less than 20% by weight) which does not allow germination to progress, however the seeds are respiring which results in a depletion of oxygen. Seeds imbibed in GA and water deteriorate faster than seeds imbibed in ABA alone or in combination with GA. The expression of TiP's (an aquaporin) correlated highly with the decline in seed germination. The results suggest conditions of anoxia result in the loss of critical proteins associated with water uptake into the tonoplast of the cell.

(b) Executive Summary

Pre-harvest sprouting is known to result in accelerated seed deterioration primarily at temperatures warmer than 30°C and a relative humidity greater than 85%. To simulate pre-harvest sprouting we imbibed seeds for 4 hours at 21°C and then dried them back to their original water content (less than 12%). Seeds were also imbibed in 25 μM gibberellic acid (GA) which is known to accelerate the germination process. We also imbibed seeds in 100 μM abscisic acid (ABA) which is a phytohormone that inhibits germination. One set of seeds was

also imbibed in a combination of 25 μMGA and 100 μMABA to determine if there was an interaction between two phytohormones. Two barley cultivars were selected; Harrington a malting barley and Dolly a feed barley. These two cultivars were selected because they are at opposite ends of the germination spectrum. Harrington tends to germinate very quickly compared to Dolly. Dried seeds from the above treatments were then placed in an incubator maintained at 30°C with a relative humidity of 85% for 1, 4, 8 and 13 weeks. Following each time interval, a batch of seeds was tested for viability using germination as an index. We also tested for the presence of a group of proteins called aquaporins that are involved in water uptake into the seed. Water uptake into the cytoplasm of the cells is regulated by these proteins. When a critical water content is attained by cells, cell division and elongation are initiated for germination. Our working hypothesis is aquaporins could serve as a marker for pre-harvest sprouting and seed viability. Dry (non-imbibed) seeds of Harrington and Dolly stored at 30°C with a 85% RH had a high germination rate for 10 and 6 weeks, respectively. By 13 weeks, germination was 20% or less for both cultivars. The number of germeable seeds of Harrington and Dolly that were imbibed in water for 4 hours declined rapidly after 6 and 4 weeks, respectively. By the 10th week, less than 5% of the seeds were germeable. The results for GA treated seeds were essentially the same as for the water treated Dolly seeds. The rate of germination of decline of Harrington seeds was somewhat slower compared to Dolly seeds. ABA alone or in combination with GA did slow the rate of seed germination decline for the first six weeks, but not thereafter. Four tonoplast membrane aquaporins (TIP) and four plasma membrane aquaporins (PIPs) were utilized in immunosorbent assays (ELISA) to determine the relationship between water transport into the cell and the viability of barley seeds subjected to 30°C, 85% RH as described above. Both TIP's and PIP's were analyzed in Harrington and

Dolly seeds, non-pre-wetted and pre-wetted for 4 hours and then dried to their original water content. There was a rapid decline in all of the TIP's but not for the PIP's of Harrington and Dolly seeds pre-wetted, dried and incubated at 30°C for 85% RH. The decrease in TIP's was strongly correlated with the loss in germination. The loss of α-TIP for seed stored at 30°C, 85% RH were determined for seeds imbibed in water, GA, ABA and a combination of ABA and GA. Seeds of both varieties had the greatest loss α-TIP if they were pre-wetted with H_20 and GA. ABA alone or in combination with GA afforded some protection for the first four weeks for Harrington and the first 13 weeks for Dolly seeds. The loss of α-TIP did not correlate strongly to the loss of germination between the loss of TiP's and the loss of seed germination for Harrington barley.

Previous reports established a close correlation between four aquaporins and pre-harvest sprouting in CPS wheats. These aquaporins can be used as markers for this trait. (See progress report-2 submitted December 30, 1999.)

Previous reports also established temperatures greater than 30°C, RH 85% resulted in rapid deterioration of barley seeds. Seeds stored at 40°C lost germination faster than seeds stored at 35°C, RH 85% which was faster than for seeds stored at 30°C. Seeds require more than 30% seed moisture content to successfully germinate. When seeds are held at 85% RH they absorb water, however, the quantity is not sufficient to allow germination to progress and the seeds essentially drown (anoxia). This was verified by storing seeds under a nitrogen atmosphere. These seeds died quicker than seeds stored in air.

To overcome seed deterioration at high temperatures and high humidity, breeders should select lines capable of overcoming anoxia.

(c) Technical Report

Seed treatment and germination

Barley seeds of Harrington (malting barley) and Dolly (feed barley) seeds harvested in 1998 from CDC, University Saskatchewan, were imbibed in water (A1), 25 μm GA (A2), 100 μm ABA (A3) and 25 μm GA+100 μm ABA (A4) on a shaker for 4 hours. The imbibed seeds then were drained and dried at 35°C in a forced-air oven for 48 hours. The dried seeds were put in cloth bags and placed in an incubator at 30°C with 85% relative humidity (RH). Non-imbibed seeds (A0) were used as a control. After one, two, four, eight and thirteen weeks of storage, 40 grams of seeds were taken from each bag to evaluate germination and aquaporin expression.

Fifty seeds were placed in a 9-cm petri dish with 3 layers of filter paper wetted with 6 ml of double-distilled water. The dishes were kept in the dark and germinated at room temperature. The number of seeds exhibiting radical emergence were recorded every day until germination was completed (generally 10 days). Germination was calculated as percentage of total seeds in each dish. Each treatment was replicated three times.

ELISA and Western analysis

Antibodies specific to four tonoplast membrane aquaporin (alpha-, delta- and gamma-TIPs, and Vm23) and four plasma membrane aquaporin (PIP1 (5.1), PIP1 (5.2), PIP2 (C), PIP1 (PAQ1)) were employed for quantification of seed aquaporin proteins by immunosorbent assays (ELISA). Non-treated and autoclaved-killed seed samples were used as controls. For each sample, 100 mg seeds were finely ground in liquid nitrogen, then homogenized with 3 ml protein extraction buffer (0.075 M potassium acetate, 0.3M NaCl, 0.1M L-arginine basic salt, 0.01 M EDTA-HCl, 0.25% triton X-100, PH 7.4), and centrifuged at 3000 rpm at 4°C for 15

minutes. Protein concentration was measured according to the Bradford bye-binding method. A 5 μg of total protein from each sample was added to a 96-well microwell plate and incubated with 100 μl coating solution (50 Mm sodium carbonate in TBS: Tris base 20 Mm, chloride 137 Mm, Hydrochloric acid 1 M) for 2 hours. The coating solution was drained and the wells washed 3 times with 100 ml TBST (5% Tween-20 in TBS). Then μl of blocking solution (1% BSA in TBS) was added and incubated for 1 hour. Following washing, 100 μl (1.5 μg/ml) of affinity-purified rabbit polyclonal anti-aquaporin antibodies were added to each cell. After incubation for 2 hours, the wells were washed 3 times. Then 100 μl (1:1500) secondary antibody (Anti-rabbit Ig, horseradish peroxidase linked whole antibody) was added. After incubation for 1 hour and 3 washes, 100 μl of colour reaction solution (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was added to each well. After 15 minutes, the plate was read in a plate reader (Kinetic microplate reader V max). All procedures were done at room temperature.

Alpha-TIP was also analyzed by the Western analysis. Total protein (10 μg) was adjusted to an equal volume (10 μl with ddH_20, 2 μg loading dye was added to each sample, and heated to 100°C for 15 minutes, cooled on ice for 10 minutes, loaded on a mini SDS-PAGE gel. After electrophoresis at 80 V for 150 minutes, the fractionated proteins were electrically transferred from the gel to a PVDF membrane (Gelman Sciences). The membrane was pre-washed in 200 ml of TBST twice, each for 15 minutes and then blocked with 200 ml of 5% milk in TBST for 1 to 2 hours. After three quick washes, the membrane was incubated with the first antibody (anti-alpha-TIP) as used for the ELISA for 2 hours. After three washes, the membrane was incubated with a second antibody (IgG-AP) for 2 hours. The membrane

was again washed 3 times. A colour developer solution (10 ml of AP buffer: 33 µl of BCIP: 66 µl of NBT) was added to the membrane. After approximately 10 minutes, the colour reaction was terminated by the addition of water.

Results and Discussion

Storage time and seed viability imbibed under different conditions

Seed viability for both varieties decreased with storage time for all imbibition treatments. The germination of control seeds (non-imbibed) did not decline as fast as imbibed seeds. The results definitely show that pre-wetted seeds of barley are very susceptible to storage conditions of high temperature and high humidity. Harrington and Dolly showed a similar germination pattern over time, except germination declined faster for the Dolly seeds (Figure 1 and Figure 2). These results indicated that pre-imbibition activated genes and enzymes associated with germination, however the activated seeds were more susceptible to adverse storage conditions than the non-imbibed seeds. ABA alone and ABA in combination of GA did not offer some protection from loss in seed germination for the first four weeks, however, little or no protection was provided thereafter.

Aquaporin expression detected by ELISA and WESTERN blot

Four different tonoplast membrane aquaporin (TIPs) and four different plasma membrane aquaporin (PIPs) antibodies were used for immunosorbent assays (ELISA) to study the relationship between aquaporin proteins and seed viability of malting and feed barleys under different imbibition conditions and different storage times. The expression of PIP and TIP aquaporin proteins in non pre-wetted Harrington barley seeds stored at 30°C at an 85% RH

for 13 weeks is shown in Figure 3. The expression of PIP proteins extracted from Harrington remained relatively constant over time. There was, however, a decrease in α-TIP over time. As a result of this finding, we quantified TIP's and PIP's in Harrington and Dolly seed pre-wetted for four hours and then dried back. The results for seeds pre-wetted for fours in water and then dried to their original water content is shown in Figures 4 and 5. In both cultivars there was a strong correlation between loss of germination and loss of aquaporin TIP proteins (Figures 1 and 2 versus Figures 4 and 5). The germination of barley seeds of cv. Harrington pre-wetted in water for four hours started to decline after four weeks of storage at 30°C, 85% RH. The concentration of TIP (Vm 23) was constant for the first four weeks but declined thereafter. Dolly seeds declined in germination after two weeks storage which corresponds to a loss of TIP (Vm 23). The most dramatic decrease in TIPs were for alpha and gamma TIP in both Harrington and Dolly seeds. To further assess the role of alpha-TIP during the decline of Harrington and Dolly seed germination, we did a more extensive study using seeds imbibed for four hours in either water, ABA, GA or a combination of ABA and GA (Figures 6 and 7). There was a rapid decline in alpha-TIP proteins for Harrington seeds imbibed in water and GA. ABA afforded some protection for the first six weeks, while a combination of ABA and GA afforded the most protection against the decline of alpha-TIP proteins. Similar results were obtained for Dolly seeds stored at 30°C in 85% RH (Figure 7). Again seeds treated with GA had the fastest decline in alpha-TIP proteins. In contrast, seeds treated with ABA alone or in combination of GA had the slowest rate in alpha-TIP decline. Western blots with Alpha-TIP showed similar trends as detected in ELISA (data not shown). Our results suggest that TIP aquaporins are sensitive to adverse conditions such as high temperatures and high relative humidity during storage. The degradation of TIP aquaporins suggests certain deteriorate if

subjected to warm temperature and high humidity. Seeds require generally more than 30% water to initiate the germination process. If seeds take up less water than this when exposed to high relative humidity, they then undergo a process known as anoxia. Our previous research using NMR analysis revealed that water is selectively absorbed in the embryo at high relative humidities. Germination of seeds exposed to a nitrogen atmosphere decline faster than seeds stored in natural oxygen conditions. In conclusion, our results suggest seed deterioration is the result of anoxia and that seeds subjected to pre-harvest wetting will deteriorate faster than non pre-wetted seed. It has been established that certain proteins are produced in seeds that reduce the effect of anoxia. Breeding programs to increase these protective proteins may overcome this storage problem of barleys exposed to high temperatures and high relative humidities. The effect of anoxia appears in part to be associated with the stability of aquaporins in the tonoplast.

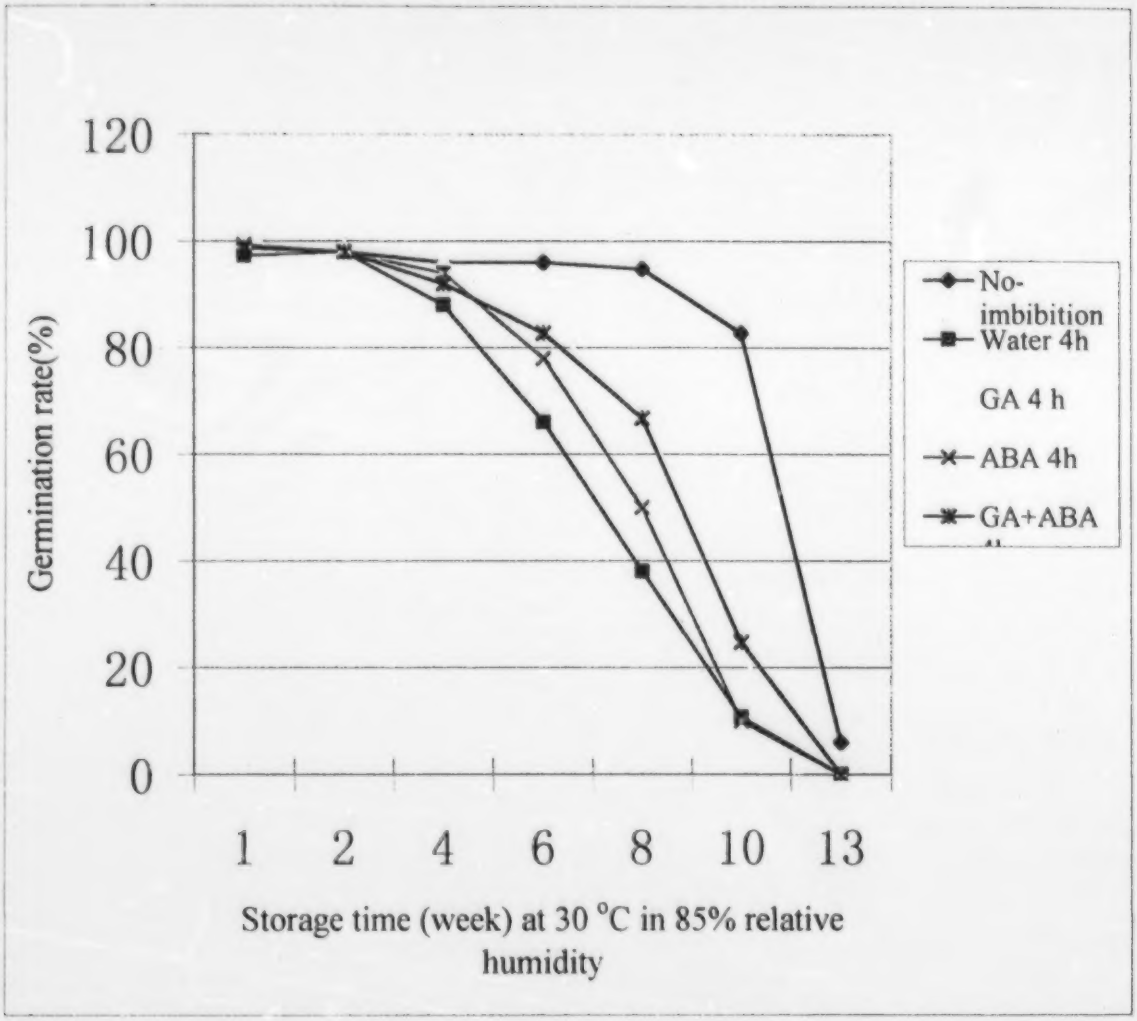

Figure 1. The effect of inbibition and storage time on Harrington seed germination

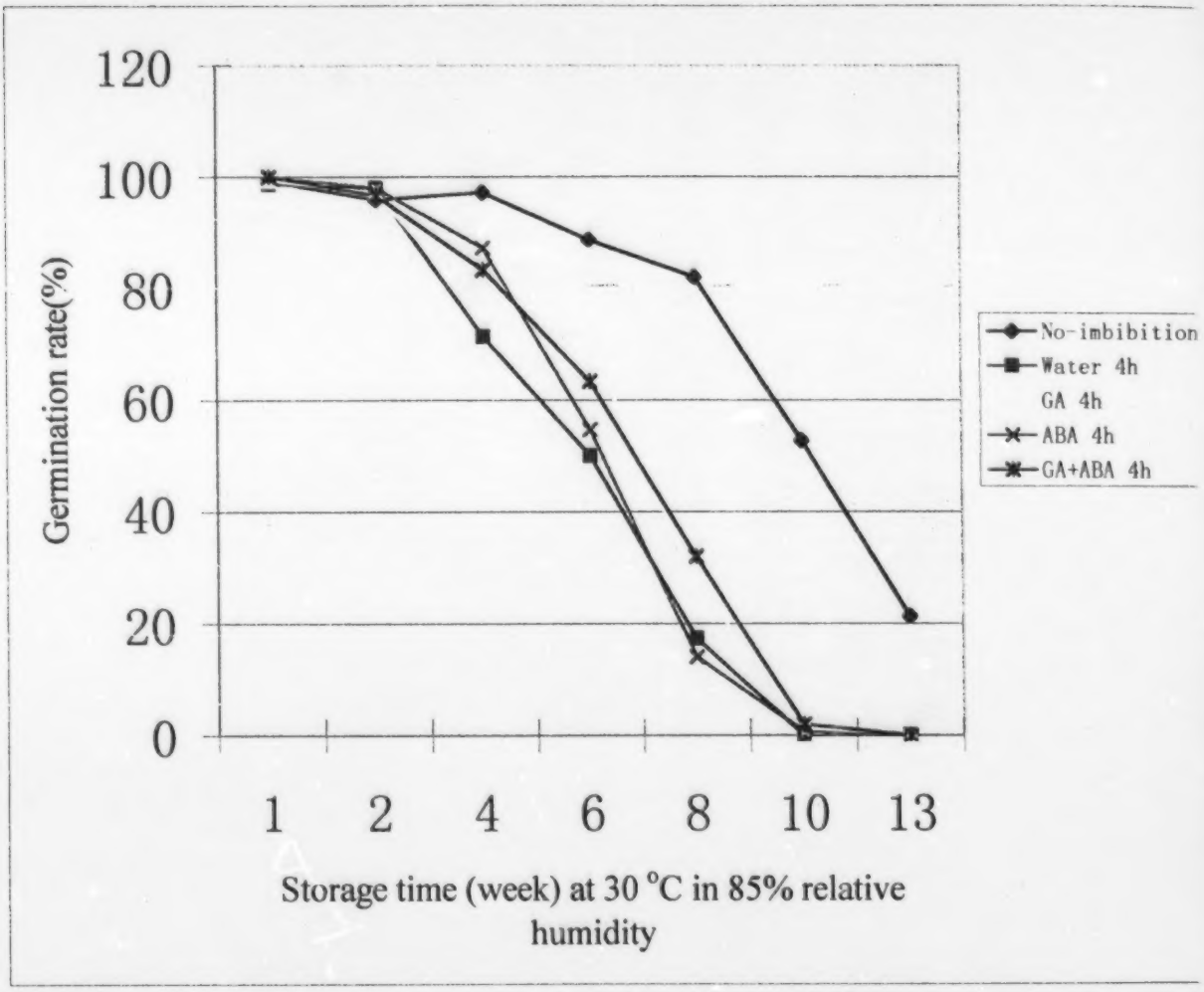

Figure 2. The effect of inbibition and storage time on Dolly seed germination

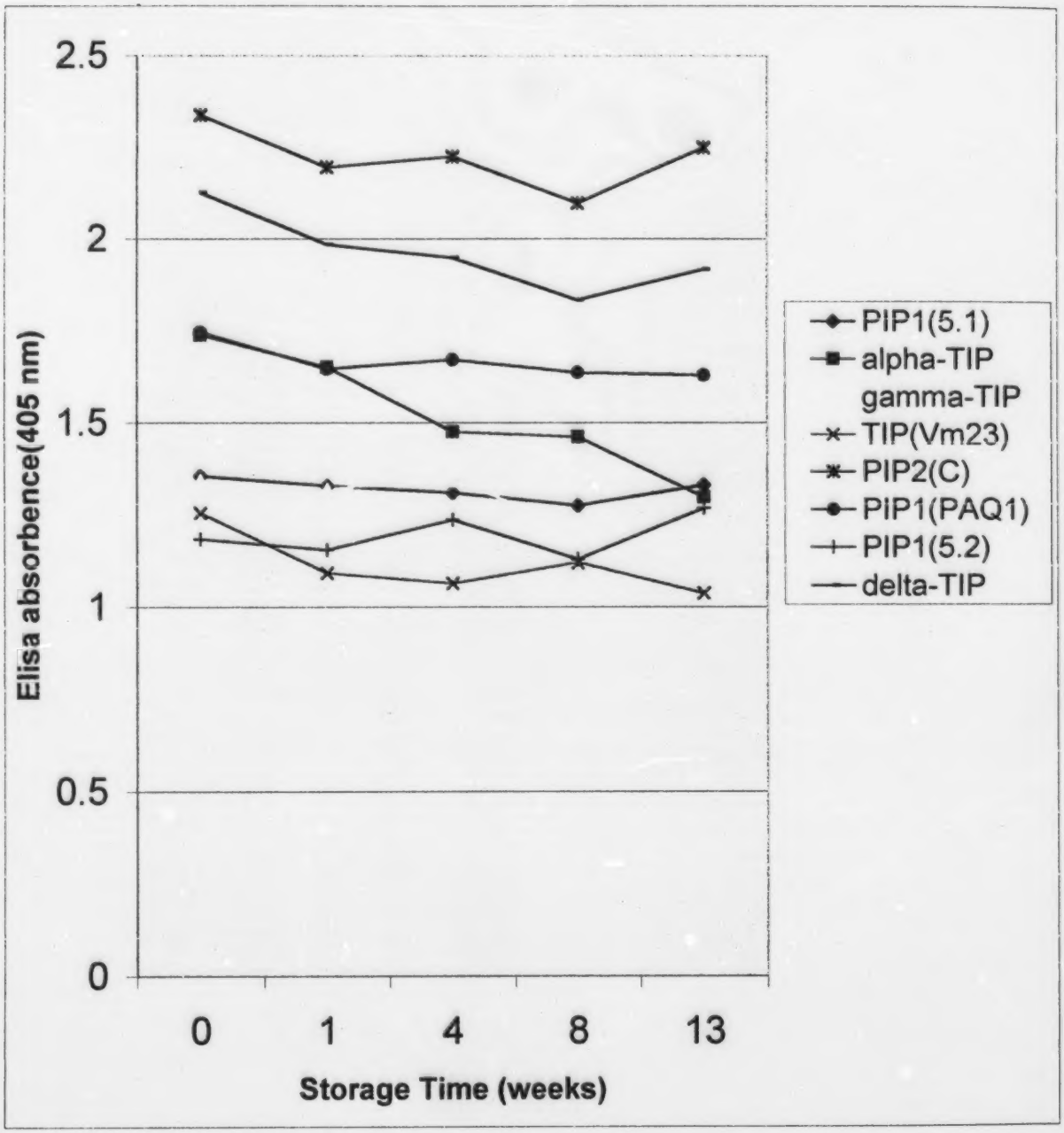

Figure 3. Immunosorbent assay (ELISA) for non-prewetted Harrington barley with different aquaporin antibodies. The barley seeds were stored at 30 °C at 85% relative humidity for 0 to 13 weeks.

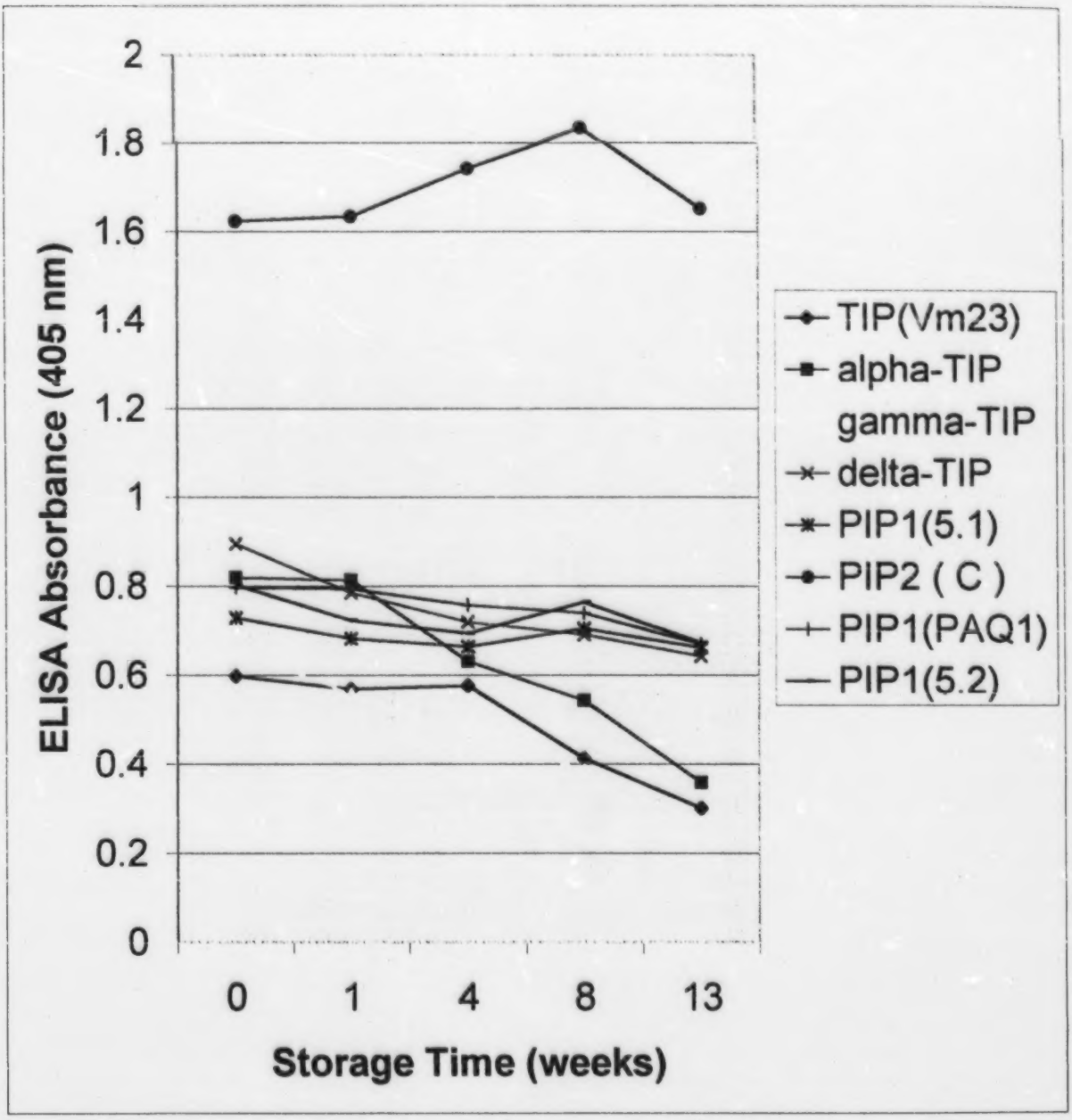

Figure 4. Immunosorbent assay (ELISA) for Harrington barley with different aquaporin antibodies. The barley seeds pre-wetted in water for 4 hours were dried to their original water content and stored at 30 °C at 85% relative humidity for 0 to 13 weeks.

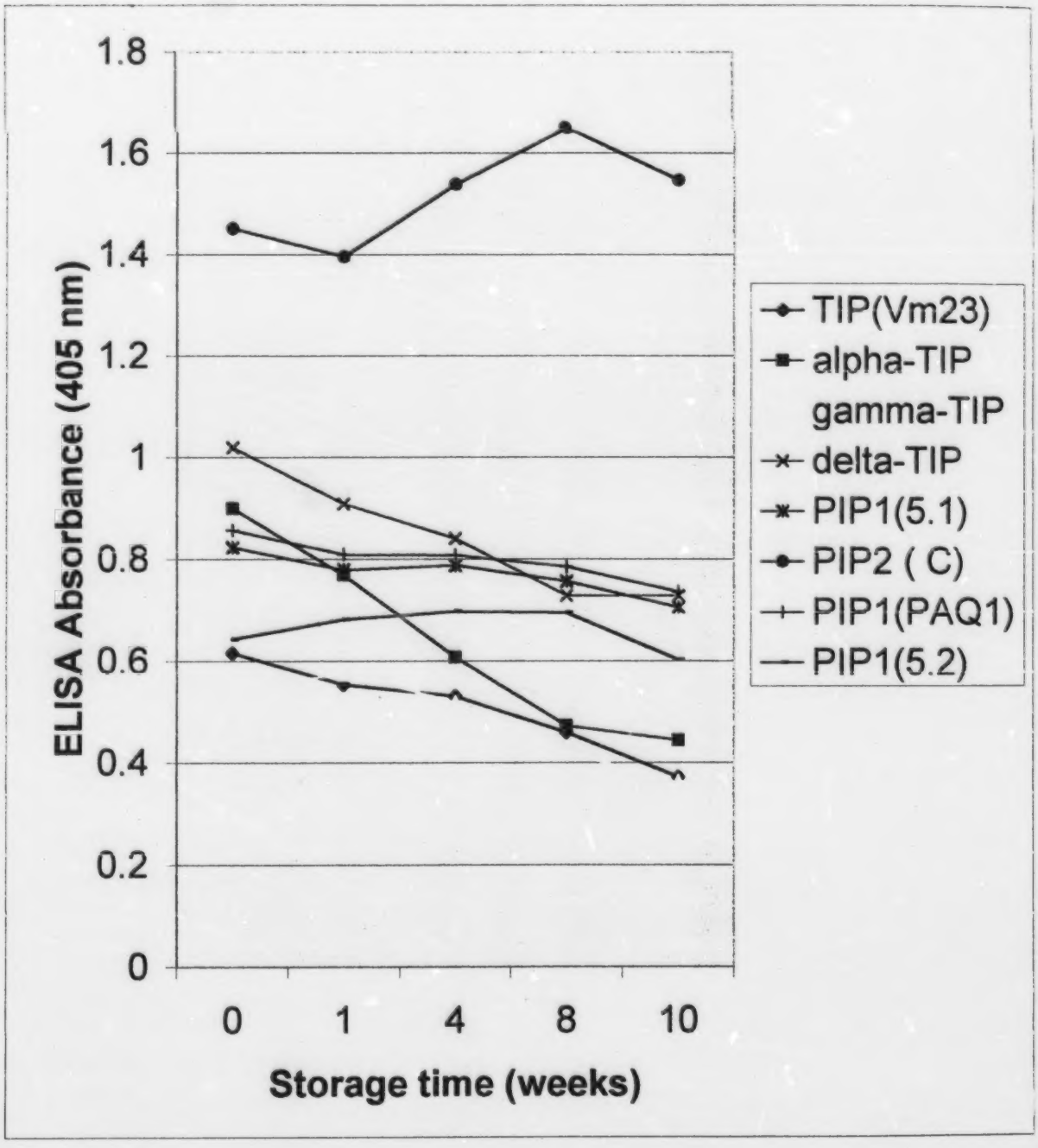

Figure 5. Immunosorbent assay (ELISA) for Dolly barley with different aquaporin antibodies. The barley seeds pre-wetted in water for 4 hours were dried to their original water content and stored at 30 °C at 85% relative humidity for 0 to 13 weeks.

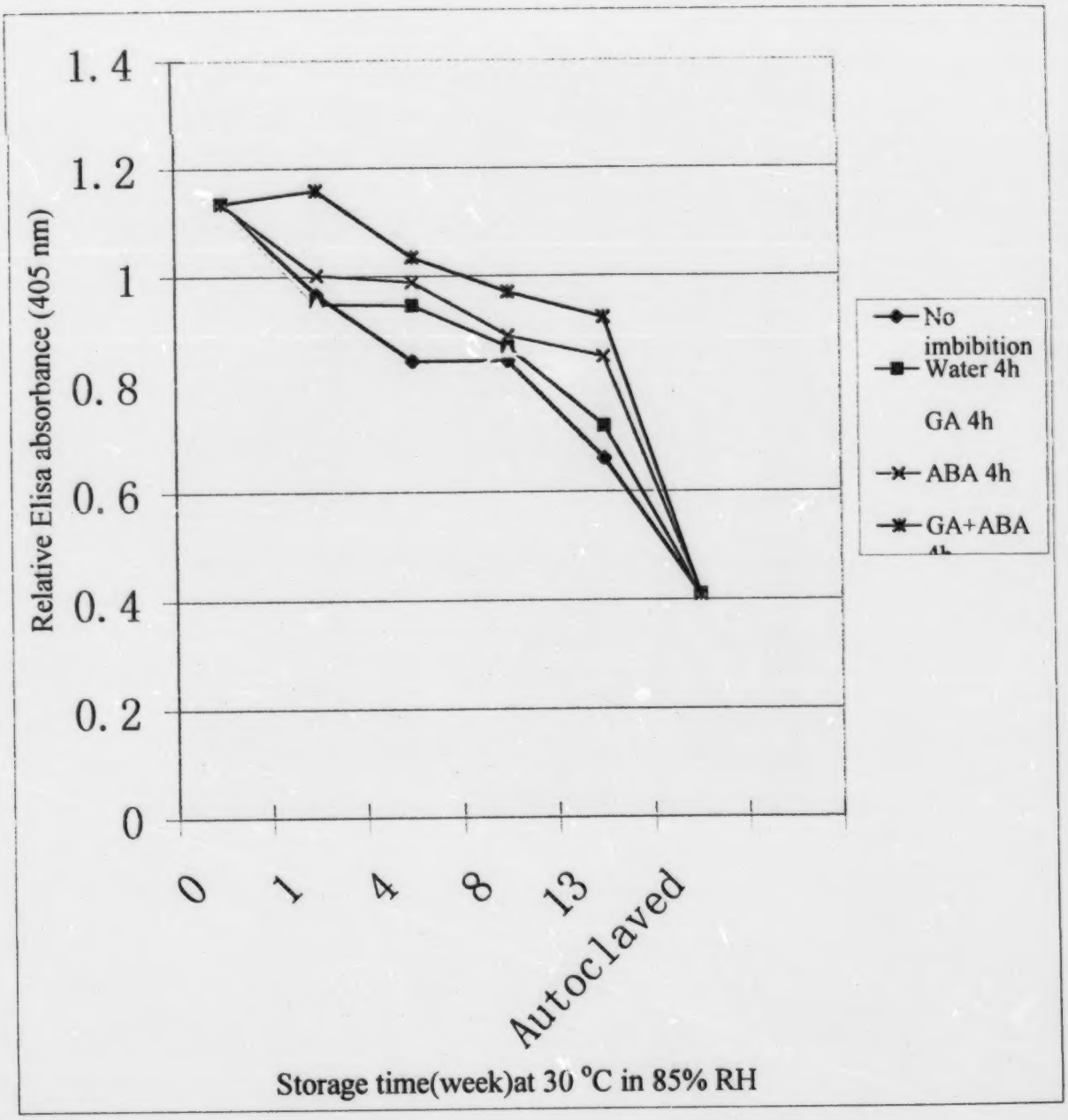

Figure 6. Immunosorbent assay (Elisa) for Harrington barley with Alpha-TIP antibodies. The barley seeds pre-wetted in water, ABA, and a combination of ABA and GA for 4 hours were dried to their original water content and stored at 30 °C at 85% relative humidity for 0 to 13 weeks.

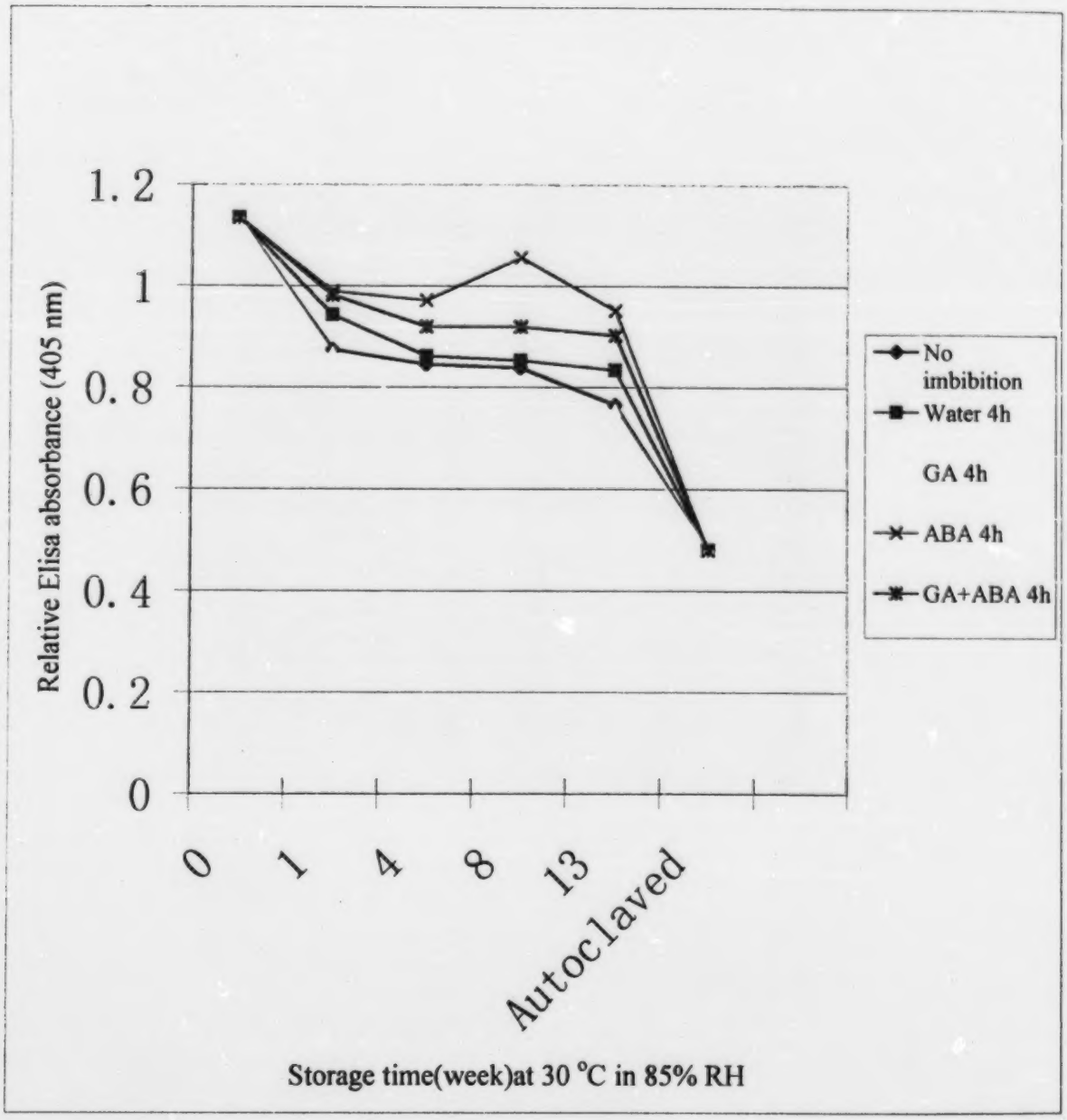

Figure 7. Immunosorbent assay (Elisa) for Dolly barley with Alpha-TIP antibodies. The barley seeds pre-wetted in water, ABA, and a combination of ABA and GA for 4 hours were dried to their original water content and stored at 30 °C at 85% relative humidity for 0 to 13 weeks.

(d) Personnel

Dr. Y.-P. Gao from April till December 31, 1999

Dr. Xiao-Qi Zhang from January 2000-June 31, 2001

Dr. X. Liu from September 2001-January 2002 (1/2 time)

(d) Equipment – none purchased over $500.00

(e) Project Developed Materials

Publication

(l) The nucleotide sequence for Ta PIP1, Ta PIP2 and Ta PIP3 were published in the EMBL, Gene Bank and NCIB Nucleotide Sequence database.

(f) Project Photos – none

(g) Acknowledgements – any publication or talks generated from the study will be acknowledged.

(h) Expense Statement – to be provided by the Business Office.